

ON PLASMA PROTEINS IN INFLAMMATION

by

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- I. Plasma protein pattern in acute infectious diseases. Scand. J. Clin. Lab. Invest. 1974. In press.
- II. Plasma protein response in experimental inflammation in the dog. Res. exp. Med. 161, 251-261, 1973.
- III. α_2 -acute phase globulin in rat serum. Purification, determination and interaction with trypsin. Biochim. Biophys. Acta, 295, 245-251, 1973.
- IV. Rat α_2 -acute phase globulin, a human α_2 -macroglobulin homologue. Interaction with plasmin and trypsin. Biochim. Biophys. Acta, 322, 62-67, 1973.
- V. Transcapillary passage of plasma proteins in experimental burns. Acta physiol. scand. 1974. In press.
- VI. A method for determining plasma proteins in inflammatory exudates absorbed by Sephadex.

INTRODUCTION

Inflammation, derived from the Latin *inflammare*, meaning to set on fire, was defined by Grawitz (1890) as the reaction of irritated and damaged, but still vital tissue. Menkin (28) defined inflammation as a defence reaction of higher animals to any injurious stimuli. It has been pointed out by others that though the inflammatory reaction is a defence mechanism, it may become harmful if not properly controlled (10). According to the general consensus of opinion, the inflammatory response consists of a local reaction and a systemic reaction. The first description of the local reaction dates back to ancient times; it was given by Celsus with the famous sentence: *Notae vero inflammationis sunt quator, rubor et tumor, cum calore et dolore*. Despite extensive investigations our knowledge of the complicated process of inflammation is still fragmentary.

The early stage of inflammation is dominated by microvascular events, *viz* opening of precapillary sphincters, vasodilatation, increase in the capillary permeability permitting increased passage of proteins from the plasma to the intercellular tissue spaces with edema as a result (18). The initially local rapid flow of blood after injury gradually decreases owing to escape of plasma through the capillary wall with consequently increased viscosity of the blood. The normally axial flow of blood corpuscles through the vessels is lost very soon after the injury, as the leukocytes begin to adhere to the vessel endothelium, a reaction which Cohnheim called a pavementing phenomenon. Then, by active amoeboid movements, leukocytes pass through the wall of venules and small veins into the extravascular tissue, a process called leukocytic emigration (19). Phagocytosis of the irritant by the leukocyte then starts. Metchnikoff was the first to describe engulfment and destruction of microorganisms by leukocytes (29).

Inflammation can be provoked by a wide variety of agents such as mechanical trauma, chemical-physical irritants, invasion by micro-organisms, cell necrosis owing to circulatory insufficiency, antigen-antibody reactions or lymphocyte-induced delayed hypersensitivity. The inflammatory reaction is nonspecific and essentially the same to the various types of stimuli (28). This was realised early and soon initiated a search for a common causal factor. It is now evident that the inflammatory reaction is brought about by several chemical mediators.

Histamine is one of the mediators in the very early phase of the inflammatory process (30,20). But the role played by histamine is transient, and the second phase of increased capillary permeability occurring 30-60 minutes after the injury is attributed to other mediators, such as serotonin and bradykinin. The action of serotonin is species-dependent and is probably only subordinate in the mediation of inflammation in man (30). Bradykinin, on the other hand, is one of the most potent vasodilators in man. The substance is a polypeptide belonging to the group referred to as kinins, all of which are activated by the action of kallikrein, a proteolytic enzyme derived from kallikreinogen, probably by the action of activated Hageman factor. Also components of the complement system, thrombin, plasmin and substances from the granulocyte membrane can activate the kallikrein-kinin system (30). Cumulative evidence has shown that the kinin system acts as a mediator in the early vasodilatation and change of capillary permeability in the inflammatory process (30). It has also recently been suggested that prostaglandins are important mediators of inflammation, but the matter is far from settled (38).

The leukocytic emigration through the capillary wall is a phenomenon entirely different from the increased capillary permeability of the vessel wall to proteins. It was realised very early by Grawitz that the movements of the leukocytes are governed by chemical substances derived

from bacteria, for example, and the phenomenon was called chemotaxis. Recent studies have identified different factors with a chemotactic effect on leukocytes. Besides many bacterial factors, some of which have been isolated, there is a factor derived from the third (C3) and the fifth (C5) components of serum complement, which causes migration of leukocytes (31,37). Also leukocytes and their degradation products can have a chemotactic effect on other leukocytes (37).

An abnormally high body temperature has long been known to be one of the systemic reactions in the inflammatory response. Granulocytosis and lymphocytosis are other systemic effects of the inflammatory process. An abnormally low concentration of serum iron and decreased iron-binding capacity are also known to accompany inflammation. In addition, the function of the pituitary and of the adrenal glands is stimulated.

The increased erythrocyte sedimentation rate (ESR) has long been used as a measure of the intensity of the systemic effect of inflammation. After the introduction of the clinical use of the ESR it was soon realised this rate was dictated mainly by the concentration of different plasma proteins. The changes occurring in the concentrations of certain plasma proteins in inflammation have been called the acute phase reaction. The finding of increased concentrations of hexose and hexosamine or of the seromucoid fraction in inflammatory processes (25,22) provided indirect measures of changes occurring in the concentration of α -globulins. An increase in the α_1 - and α_2 -globulin fractions in the electrophoretic protein pattern denoted an acute inflammation (17,4), a pattern sometimes called that of an "active" acute inflammation. When also the γ -globulin fraction was increased, the pattern was denoted "chronic" inflammation.

Determinations of specific plasma proteins have since revealed which proteins increase or decrease in

the acute phase reaction (8,21). Despite extensive investigation, it is still mainly unknown what functions the individual proteins have in the inflammatory process. Even when the normal function of a given protein is known, one cannot be sure that an increase in it in inflammation is due to an increased requirement. An increased synthesis of it might be a passive accompaniment of increased synthesis of some other proteins, provided they are regulated by a common mechanism.

α_1 -antitrypsin and antichymotrypsin increase in concentration in most inflammatory reactions. The two proteins together with α_2 -macroglobulin are known to bind and inhibit proteolytic enzymes (27,36). Several such enzymes, such as leukocytic proteases, are released in inflammation (5). It is reasonable to assume that increased synthesis of the inhibitors of the enzymes would be of beneficial in inflammation. α_2 -macroglobulin does not increase in inflammation in man, but in other species its homologue reacts like an acute phase protein (14,15).

It is known that orosomucoid, which increases markedly in concentration in inflammation, binds progesterone and, to some extent, also other steroid hormones, such as cortisol (11). It is not known whether this property of orosomucoid also serves a physiological function. But if it does, the increased concentration of corticosteroid hormones in inflammation would perhaps require a large amount of their carrier.

In inflammation synthesis of haptoglobin is increased (2). This increase may, however, be masked by a coexisting increase in the elimination of hemoglobin. The hemoglobin binding function of haptoglobin is known. But it is unsettled why this capacity should be increased in inflammatory processes. The course of inflammation is not known to be worse in patients with hemolytic disorders without a measurable concentration of haptoglobin. One might imagine that the origin of the increase in synthesis of haptoglobin

dates back to the time when malaria infection with severe hemolysis was a common cause of inflammation. Malaria has surely governed the evolution of the prevalence of hemoglobin-S (1).

C-reactive protein (CRP) increases rapidly and considerably in inflammation, which might suggest that it plays an important role in the inflammatory defence, especially since CRP stimulates phagocytosis of bacteria (23). The third component of complement (C3 or β_{1C} -globulin) increases in concentration in inflammation. It is now known that complement participates in the inflammatory reaction in several various ways (31). The histamine-induced increase in capillary permeability early in the inflammatory process is caused by C3 and C5 (also called anaphylatoxin), as is the chemotactic activity on emigration of leukocytes.

The increase in the concentration of fibrinogen in the plasma after injury is associated with an even larger increase in its synthesis (24). It appears reasonable to suppose that the requirement of fibrinogen is increased in inflammation, as plasmin is often activated with consequent intravascular fibrinogenolysis, or fibrinogen may be deposited around the lesions (9). Furthermore, injury is often combined with bleeding, and then the requirement of proteins taking part in the blood coagulation might be increased.

It is now evident that ceruloplasmin catalyses the oxidation of ferrous ions, a process occurring in several stages of the iron metabolism (34). But the significance of the increased concentration of ceruloplasmin in inflammation is unknown.

The plasma protein response has been studied extensively in many different types of inflammation, such as that in myocardial infarction, after operation and in ulcerative colitis (21,7,39). Yet no specific patterns of different types of inflammation have hitherto been demonstrated. The concentration of many of the acute phase

reactants are influenced also by factors other than inflammation, such as α_1 -antitrypsin by the occurrence of the gene for α_1 -antitrypsin deficiency or by the influence of estrogen hormones. The latter is also valid for ceruloplasmin, whereas the concentration of haptoglobin depends on the rate of the red cell turnover. Complement activation lowers the concentrations of C3 and C4. The influence of factors other than inflammation explains why so many different proteins usually are studied in investigations of the inflammatory reaction. Infection is a type of inflammation in which specific proteins have not been studied in detail. One of the purposes of the present investigation was to try to find a reaction common to infections and possibly of practical diagnostic value. Another purpose of the study was to try to find differences in the acute phase reaction between various species as a possible aid in understanding which of the proteins might be of the greatest significance in the inflammatory response.

PRESENT INVESTIGATION

Acute phase reaction in infectious diseases (I)

The response of the acute phase reactants was studied in peritonitis, influenza A and serous meningitis. The general pattern with a marked increase in the concentration of haptoglobin, orosomucoid, fibrinogen, antichymotrypsin, α_1 -antitrypsin and C-reactive protein was similar in all three diseases, which also is in accord with what is found in other inflammatory processes studied (21,3). Yet the changes in peritonitis were more pronounced than in the other two diseases, which might be explained by a larger local tissue destruction or by the difference in etiology, *i.e.* bacterial or viral. The difference in the levels of the concentration of the acute

phase proteins in peritonitis and the other two diseases were not so marked as to be of any differential diagnostic aid in a given case.

As for complement C3 and C4, there was a difference between the diseases, in that C3 was not elevated and C4 only moderately elevated in influenza A. In the other two diseases C3 and C4 were markedly increased, as they usually are in inflammation.

The fact that half of the patients with influenza A had lower concentrations of C3 during illness and otherwise suggests coexisting activation and elimination of complement. This explanation might be valid also for the small increase of C4. As different types of influenza A have been prevalent in recent years it is very probable that many patients had had the disease before and therefore had complement-binding antibodies to influenza virus. On re-infection an antigen-antibody reaction occurred and consumed complement.

Acute phase reaction in the dog (II)

The inflammatory reaction of plasma protein was studied and found to be similar in several types of injury in dogs - bacterial arthritis, turpentine injury, thrombophlebitis, proctitis and cholecystectomy. The response of most of the proteins in dogs did not differ from that in man. Thus, there was an increase of C-reactive protein, haptoglobin, fibrinogen, β_{1C} -globulin and ceruloplasmin with the same lapse as in man. However, the relative increase in the concentration of orosomucoid was more pronounced than the increase in any other acute phase reactant (except CRP), and it was much more marked than in human inflammation. The reaction of α_1 -antitrypsin differed also from that in man, in that no change in the concentration occurred.

Since the pronounced increase of orosomucoid in inflammation is the largest of all the proteins (except

CRP), orosomucoid might play an important role in inflammation. This is further supported by the fact that, judging from preliminary results of experimental inflammation in dogs with only very mild injury, CRP and orosomucoid are the only acute phase reactants that increase.

Noting is known with certainty about the role played by α_1 -antitrypsin in the inflammatory reaction in the dog, until its synthesis in inflammation has been cleared up. An increased synthesis might be balanced by a larger catabolism, *i.e.* after binding to proteolytic enzymes, and thus giving unchanged concentration of α_1 -antitrypsin in the blood. But it seems unlikely that any existing increased synthesis of α_1 -antitrypsin should balance the catabolism exactly in all animals. One might expect that in some of them synthesis would preponderate over catabolism and in some of them *vice versa*, with a higher or lower concentration as a result. This together with the fact that α_1 -antitrypsin is not regularly elevated in inflammation in man (6) and is not normally correlated in concentration with the other acute phase reactants in man (13), might suggest that α_1 -antitrypsin does not play an essential role in inflammation. Thus the large increase of orosomucoid in the dog may suggest the importance of orosomucoid in inflammation also in man and the absence of increase in α_1 -antitrypsin in the dog might indicate that also in man α_1 -antitrypsin is of less importance in inflammation.

α_2 -acute phase globulin in the rat, a human α_2 -macroglobulin homologue (III,IV)

A protein called α_2 -acute phase globulin (α_2 -AP globulin) appears in plasma of rats after injury (15). It was shown that the protein is also normally present in a mean concentration of 40 mg/l (III). To be able to

quantitate the protein in serum, α_2 -AP globulin had first to be purified. This was done by a three-stage procedure with ammonium sulphate precipitation, DEAE chromatography and Sephadex G200 gel filtration (III).

As α_2 -AP globulin is a macroglobulin and immunologically related to α -macroglobulins of several mammals (16) one would expect it to bind trypsin and plasmin as do other α -macroglobulins (27,35). Crossed immunoelectrophoresis followed by autoradiography of mixtures of rat sera and ^{125}I -labelled bovine trypsin and rat sera and ^{125}I -labelled human plasmin was used to show binding of trypsin and plasmin to α_2 -AP globulin (III and IV). The way in which trypsin is bound to α_2 -AP globulin is similar to the way in which trypsin is bound to human α_2 -macroglobulin, as shown by the remaining activity of the complexed trypsin on a low molecular weight substrate (benzoyl-DL-arginine-*p*-nitroanilide·HCl, BAPNA) (IV). Together with the earlier known immunological relationship the findings strongly indicated that α_2 -AP globulin is a true homologue of human α_2 -macroglobulin.

Like α_2 -AP-globulin in the rat, α_1 -macroglobulin in the rabbit is known to be an acute phase reactant (14). Yet the relative increase in injury of the former is much larger than that in the latter. The concentration of α_2 -macroglobulin in man is unchanged after injury (8,21). But the role of the α_2 -macroglobulin as an acute phase reactant in man cannot be definitively decided until the rate of synthesis of the protein after injury is known. An increase in synthesis in association with increased binding of the protein to proteolytic enzymes would mask the role of α_2 -macroglobulin in the inflammatory process. However, it is known that the increase in the rate of synthesis of α_2 -macroglobulin after streptokinase treatment is very low (32). The unchanged concentration of α_2 -macroglobulin in inflammation in man therefore argues against participation of the pro-

tein in the inflammatory reaction in man.

Leakage of plasma proteins in thermal injury (V)

To elucidate the changes in capillary permeability in thermal injury the concentrations of plasma proteins with different molecular weights were measured in lymph from dogs subjected to burns. Though the molecular weights of the canine proteins are not known, they are assumably about the same as those of the corresponding human proteins. The lymph concentration/plasma concentration ratios were markedly increased for orosomucoid, α_1 -antitrypsin, albumin, haptoglobin, IgG and β_{1C} -globulin after burn. There was no significant change in the lymph/plasma ratio of α_2 -macroglobulin (V).

The increase, after thermal injury, in the lymph/plasma ratio of those proteins with molecular weights up to 300,000 was interpreted as a manifestation of increased capillary permeability. The mean of the relative increase of the ratios showed a tendency to increase with the molecular weight of the protein. This tendency was what might have been expected. Assuming extreme injury of the capillary membrane with consequent free passage of proteins of all molecular sizes, the lymph/plasma ratio would be 1.0 for all proteins. The proteins with the highest molecular weights, starting with the lowest values of ratio, will naturally show the largest relative increase in the ratio.

The absence of change in lymph/plasma ratio of α_2 -macroglobulin could mean either that the capillary permeability to the large molecule of α_2 -macroglobulin is not increased, or that the permeability is increased, but that α_2 -macroglobulin is being "consumed" in the interstitial tissue by being bound to proteolytic enzymes released. It is known that the α_2 -macroglobulin-protease complexes are eliminated very quickly by the reticulo-endothelial system (33).

In the investigation of permeability to macromolecules of the capillary wall it is usual to deal with the relationship between the concentration outside the capillary system (in interstitial fluid or in lymph) and the one inside (26). The quotient, here called lymph/plasma ratio (sometimes called C_L/C_P) is taken as a measure of the capillary permeability when comparing molecules of different molecular weights. But when regarding the capillary permeability from a functional point of view, another quotient would be more relevant. The function of the capillary wall in this connection might be to maintain a concentration gradient across the membrane. The difference between the concentration in plasma and in lymph in relation to the concentration in plasma ($\frac{C_P - C_L}{C_P}$) might therefore be regarded as an expression of the ability of the capillary membrane to maintain the gradient. $\frac{C_P - C_L}{C_P}$ ratios reflect the capillary permeability more truly, especially when changes in it are studied. After thermal injury causing an increase in capillary permeability to plasma proteins the $\frac{C_P - C_L}{C_P}$ ratios will fall. It is immaterial which of the two expressions of ratio is used when studying increased permeability to proteins with lymph/plasma ratio about 0.5, since the relative change in the ratio will be the same. But when using the lymph/plasma ratio of large molecules (with a low lymph/plasma ratio) the relative increase in this ratio will be overestimated as compared with the relative decrease of $\frac{C_P - C_L}{C_P}$ ratio. On the other hand, small protein molecules with a high lymph/plasma ratio will result in underestimation of the relative change in the lymph/plasma ratio.

The $\frac{C_P - C_L}{C_P}$ ratios are given for comparison with the lymph/plasma ratios of the present study (V) in table 1. After thermal injury there was a significant decrease in the $\frac{C_P - C_L}{C_P}$ ratio of all proteins studied, except α_2 -macro-

	orosomucoid	α_1 -anti-trypsin	albumin	haptoglobin	IgG	β_{1C} -globulin	α_2 -macro-globulin
Lymph/plasma ratio before scalding	0.53 (0.44-0.62)	0.50 (0.38-0.68)	0.47 (0.40-0.56)	0.42 (0.30-0.52)	0.40 (0.33-0.47)	0.39 (0.32-0.44)	0.31 (0.24-0.39)
Lymph/plasma ratio 2 hrs after scalding	0.76 (0.61-0.82)	0.76 (0.67-0.81)	0.73 (0.66-0.84)	0.64 (0.52-0.72)	0.62 (0.57-0.66)	0.63 (0.58-0.67)	0.35 (0.28-0.44)
Percent increase in lymph/plasma ratio	43 (31-80)	48 (18-105)	52 (26-98)	51 (18-97)	54 (30-83)	61 (38-98)	6 (-13- +79)
$\frac{C_P - C_L}{C_P}$ ratio before scalding	0.47 (0.38-0.56)	0.50 (0.32-0.62)	0.53 (0.44-0.60)	0.58 (0.48-0.70)	0.60 (0.53-0.67)	0.61 (0.56-0.68)	0.69 (0.61-0.76)
$\frac{C_P - C_L}{C_P}$ ratio 2 hrs after scalding	0.24 (0.18-0.39)	0.24 (0.19-0.33)	0.27 (0.16-0.34)	0.36 (0.28-0.48)	0.38 (0.34-0.43)	0.37 (0.33-0.42)	0.65 (0.56-0.72)
Percent decrease in $\frac{C_P - C_L}{C_P}$ ratio	48 (27-62)	48 (32-68)	47 (28-64)	36 (21-54)	36 (27-46)	39 (29-50)	2.1 (-7-+26)

Table 1. Lymph/plasma ratio and $\frac{C_P - C_L}{C_P}$ ratio of various proteins before and after scalding.

globulin. The range of the relative decrease in $\frac{C_P - C_L}{C_P}$ ratios of different proteins was obviously narrower than the corresponding relative increase in the lymph/plasma ratio. The mean decrease in $\frac{C_P - C_L}{C_P}$ ratio of the proteins did not differ significantly. This means that the damage to the capillary membrane with consequent lower retention of macromolecules is the same within the range of molecular weights studied.

Plasma proteins in inflammatory exudates (VI)

A method was devised for collecting exudate from weeping sores or burns by absorption with Sephadex. It was soon realised that such absorption also had a remarkable therapeutic effect, which will be considered elsewhere (12). To determine the concentration of various substances in the exudate it was necessary to convert the fairly dry Sephadex gels into a liquid. With Sephadex G200 this was done by ultrasonic disintegration of the gel after it had swelled in ^3H -water. The isotope was used to enable determination of the strength of the dilution. But with Sephadex G25 it was not possible to disintegrate the beads by sonication. These gels were therefore swelled in a mixture of two isotopes, namely ^3H -water to permit estimation of the dilution of water and ^{131}I -labelled albumin to estimate the volume outside the Sephadex G25 beads. The mean error of the method was 2.4 % when Sephadex G200 was used and 3.2 % when Sephadex G25 used. The plasma proteins were determined by electroimmunoassay to exemplify an application of the method (VI).

Actually the determination of the dilution when swelling Sephadex G200 could be done without ^3H -water. By simply weighing the gels before and after they had swelled, the dilution factor could be estimated. But this method proved

less accurate than the one with ^3H -water, when the gels were dry, and they usually were, and had to be diluted considerably.

The method of collecting exudate by Sephadex and estimating the volume of exudate by an isotope-dilution technique seems suitable also for determining constituents of inflammatory exudate other than plasma proteins. It must, however, then be checked that Sephadex does not interfere with the substance in question.

SUMMARY

The following observations of interest were made in the present investigation of plasma proteins in inflammation.

1. The response of those plasma proteins designated acute phase reactants was studied in peritonitis, serous meningoencephalitis and influenza A in man. The reaction in acute infections did not differ from that known in other inflammatory conditions in man.

2. The acute phase reaction in the dog differed from that in man in that the increase in concentration of orosomucoid was larger and that no increase occurred in α_1 -antitrypsin.

3. The α_2 -acute phase globulin of the rat was purified. It was shown to occur also normally in the rat in a concentration of 40 mg/l. The protein bound plasmin and trypsin and furthermore the complex with trypsin was still active on a low molecular weight substrate. The findings strongly indicate that α_2 -acute phase globulin of the rat is a homologue of human α_2 -macroglobulin.

4. The changes of capillary permeability in burns were studied by determining plasma proteins in lymph in experimental burns in dogs. There was an increase of concentration in lymph of those proteins with a molecular weight up to 300,000 indicating an increased capillary permeability. But there was no rise in the concentration of α_2 -macroglobulin (MW 820,000) in the lymph after burn, indicating unchanged capillary permeability to the very large molecules. However binding of α_2 -macroglobulin to proteolytic enzymes followed by rapid elimination of the complex could not be excluded.

5. A method devised for collecting inflammatory exudate from weeping sores and burns by Sephadex followed by an isotope-dilution technique to determine the amount of exudate absorbed seems suitable also for determining various constituents of exudates, such as chemical mediators of inflammation.

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